

The carboxylation of prothrombin precursors in a bovine liver cell-free system

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THE CARBOXYLATION OF PROTHROMBIN PRECURSORS IN A BOVINE LIVER CELL-FREE SYSTEM

C. VERMEER, M. DE METZ, B. A. M. SOUTE and
H. C. HEMKER

Department of Biochemistry, State University of Limburg, Maastricht,
The Netherlands

ABSTRACT

Vitamin K-dependent carboxylation was studied in partly purified bovine liver enzyme systems (carboxylase). As there is no absolute requirement for an exogenous substrate, the enzyme preparations most probably contain endogenous substrates. Carboxylase was prepared, either from normal cows or from cows that had been anticoagulated with a vitamin K antagonist. In carboxylase from anticoagulated cows the carboxylation was 20-fold enhanced as compared to carboxylase obtained from normal cows. This is probably due to increased quantities of endogenous substrate as in the presence of an excess of exogenous substrate both carboxylase preparations incorporate $^{14}\text{CO}_2$ equally well.

KEYWORDS

Vitamin K, carboxylase, prothrombin synthesis, anticoagulation, γ -carboxy-glutamic acid, warfarin.

INTRODUCTION

During anticoagulation with vitamin K-antagonists, the blood plasma level of the four vitamin K-dependent coagulation factors decreases, and an abnormal form of these factors arises in man and in the cow (Hemker and coworkers, 1963; Ganrot and Nilehn, 1968). It was shown that the normal clotting factors contained near the N-terminal region ten to twelve glutamic acid (glu) residues which had been carboxylated at the γ -position. These γ -carboxy glutamic acid (gla) residues turned out to be normal glu residues in the abnormal clotting factors (Stenflo and coworkers, 1974). The latter factors were designated therefore as decarboxy factors. It seemed evident to conclude that vitamin K mediates in an up to then unknown carboxylation reaction.

After this discovery a number of people developed liver cell-free systems from the warfarin treated rat, in which $^{14}\text{CO}_2$ was incorporated into TCA precipitable material and in a vitamin K-dependent way (Suttie and coworkers, 1979). As prothrombin precursors accumulate in the rat liver during warfarin treatment, these enzyme systems (carboxylase) contained substantial amounts of the natural substrate (Carlisle and coworkers, 1975). In our lab we prepared carboxylase from cow liver. As the decarboxy factors are released in the blood during anticoagulation, it seemed probable that hardly any prothrombin precursors are present in these carboxylase preparations. Therefore, we studied whether purified decarboxy prothrombin might serve as a substrate for the vitamin K-dependent carboxylation.

MATERIALS AND METHODS

Chemicals

Vitamin K₁ was obtained from Hoffmann-La Roche, Switzerland, and warfarin and subtilisin from Sigma, USA. NaH¹⁴CO₃ (60 mCi/mmol) was purchased by The Radiochemical Centre, Amersham, England. All other chemicals were obtained from Merck, G.F.R.

Preparation of decarboxy prothrombin

Cows (300 - 400 kg) were anticoagulated for 1 week with 300 mg of warfarin daily. After this period, they were either sacrificed for the production of carboxylase (see below) or 5 l of blood were withdrawn for the production of decarboxy prothrombin. Decarboxy prothrombin was purified as described by Vermeer and coworkers (1978 a).

Preparation of carboxylase

Crude microsomes were prepared as described earlier (Vermeer and coworkers, 1978 b), and extracted with 0.5 % triton X-100 in 1 M KCl, 0.02 M tris-HCl, pH 7.8. Insoluble material was removed by centrifugation (1½ h, 150,000 x g) and the supernatant was designated as carboxylase. The solution was diluted in such a way, that 1 ml of carboxylase was equivalent to 1 g of liver. Carboxylase was prepared from normal cows as well as from anticoagulated cows in a similar way.

Measurement of carboxylase

Carboxylase activity was measured in reaction mixtures of 0.5 ml which contained 0.2 ml solubilized carboxylase, 0.3 M NaCl, 0.05 M tris-HCl, pH 8.0, 0.2 % triton X-100, 20 µCi NaH¹⁴CO₃ and exogenous substrate as indicated. The mixtures were incubated at 25°C and carboxylation was started by adding 10 µM chemically reduced vitamin K₁. The reaction was stopped by precipitation with TCA, followed by centrifugation. The precipitates were dissolved in 1 ml 1 M NaOH and reprecipitated with TCA (3 times). Finally, the precipitates were dissolved and counted.

Small peptides, which were sometimes used as substrates for carboxylase, do not precipitate in TCA. In that case traces of unbound label were removed from the TCA supernatant by vacuum extraction over KOH, whereafter the samples were counted.

RESULTS AND DISCUSSION

The incorporation of ¹⁴CO₂ in endogenous substrate was measured after several periods of time in carboxylase prepared from normal and from anticoagulated cows (Fig. 1 A). It turned out, that in both systems carboxylation could be demonstrated which means that both systems contain a substrate for carboxylase. Moreover, carboxylase obtained from anticoagulated cows exhibited a 20-fold increase of ¹⁴CO₂ incorporation as compared to normal carboxylase. Analysis of the reaction product on polyacrylamide gels in SDS showed, that about 70% of the label had been incorporated in a protein with a M.W. of 70,000 D (± 2000), which is the M.W. of prothrombin. When thrombin was added to the samples before electrophoresis, the 70,000 D peak had disappeared and all of the label was recovered in a new peak at 24,000 D (± 1000). This is the M.W. of fragment-1, the N-terminal fragment that is cleaved from prothrombin by thrombin. Therefore, we believe that most of the endogenous substrate is a prothrombin precursor.

We also examined whether purified decarboxy prothrombin could serve as an exogenous substrate (Fig. 1 B). In these experiments, we used carboxylase from normal cows, because it probably contains less endogenous substrate than carboxylase from anticoagulated cows does. From these experiments it turned out:

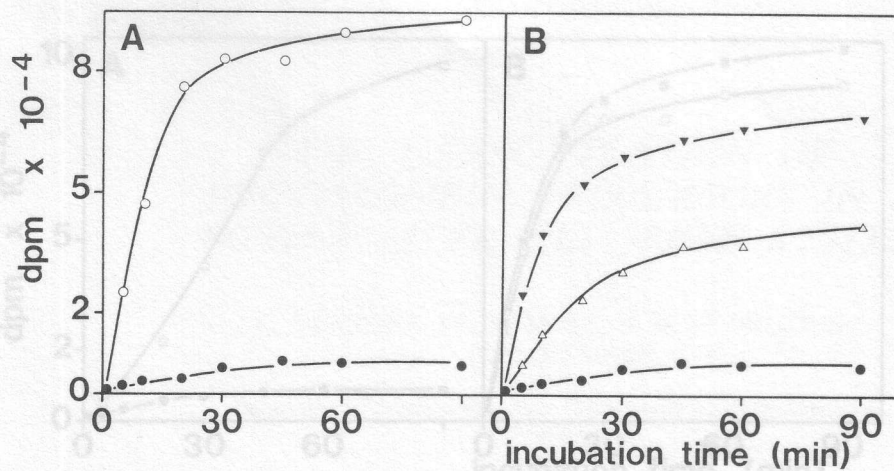


Fig. 1 A. Time course of carboxylase obtained from normal cows (●—●) and that obtained from anticoagulated cows (○—○).

Fig. 1 B. Carboxylation in the presence of various concentrations of decarboxy prothrombin: ▼—▼ 0.3 mM; △—△ 0.07 mM; ●—● 0 mM. The experiments were performed with carboxylase from normal cows.

- that adding decarboxy prothrombin to the reaction mixtures strongly enhances the carboxylation reaction and
- that in the presence of an excess of substrate, carboxylase from normal cows is equally well active as carboxylase from anticoagulated cows.

However, as a high K_M was measured for decarboxy prothrombin (about 0.5 mM), the latter could not be looked upon as a good substrate for carboxylase.

A better substrate was obtained when decarboxy prothrombin was cleaved with Sepharose-bound subtilisin. This cleavage resulted in a fragment of 8000 D (± 1000) with a K_M of about 0.03 mM. Therefore, this fragment is a much better substrate for carboxylase. As is shown in fig. 2, only carboxylase from normal cows can be stimulated by this low molecular weight substrate. The most plausible interpretation of these results is, that similar amounts of carboxylase are present in liver tissue from normal cows and from anticoagulated ones, and that during anticoagulation the endogenous substrate is not completely released as decarboxy prothrombin, but partly accumulates in the liver. Moreover, it seems that decarboxy prothrombin as it is obtained from blood plasma, is not a good substrate for carboxylase. A better substrate is obtained after limited proteolytic degradation. Speculating about this phenomenon one might expect that in vivo the carboxylation reaction is followed by a second modification (e.g. a glycosylation or limited proteolysis at the N-terminal side). When the carboxylation is temporarily blocked by vitamin K-antagonists, this last modification is still carried out on the decarboxy factors, which then enter the blood stream. This modification hampers the in vitro carboxylation in our system.

521 - 523.

Vermeer, C., B.J.H. Soute and H.C. Bunker (1978 b). In vitro prothrombin synthesis from a purified precursor prothrombin. II. Partial purification of bovine carboxylase. *Biochim. Biophys. Acta*, 523, 554-565.

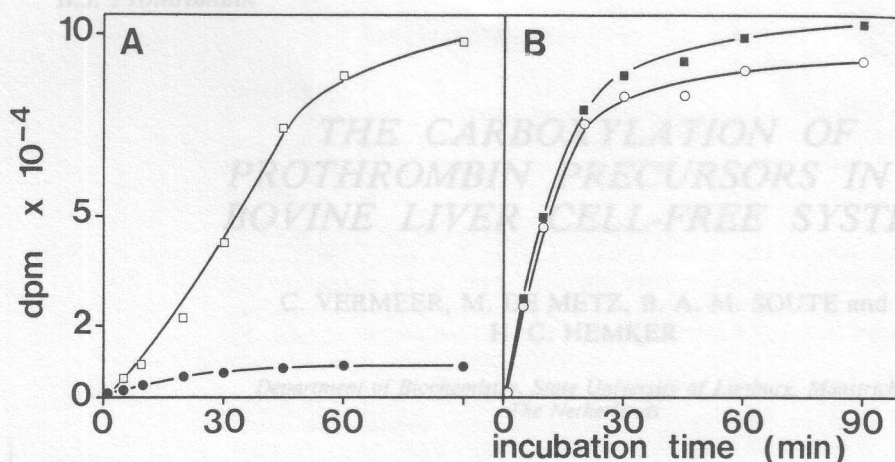


Fig. 2 A. Time-course of the carboxylation reaction in a normal cow liver system in the absence (●—●) and presence (□—□) of subtilisin-fragmented decarboxy prothrombin.

Fig. 2 B. Time-course of the carboxylation reaction in an anticoagulated cow liver system in the absence (○—○) and presence (■—■) of subtilisin-fragmented decarboxy prothrombin.

Both figures represent the total amount of dpm found in the TCA precipitate and in the TCA supernatant.

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